Supplementary Information of
A bivalent inhibitor against TDRD3 to suppress phase separation of methylated G3BP1

Authors

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Materials and Methods

Cloning, protein expression, and purification

G3BP1 fragments (residues 327-466) were PCR-amplified from a human brain cDNA library and were then cloned into the pET-28a vector. The constructs were then transformed into *Escherichia coli* BL21.

Proteins were overexpressed at 37 °C after induction with 0.5 mM IPTG for 6 hours. Bacterial cells were harvested by centrifugation followed by resuspension in lysis buffer (2 M NaCl and 20 mM NaH_2PO_4, pH 6.5). After sonication, lysates were centrifuged, and the supernatants were loaded onto nickel affinity columns (QIAGEN, Shanghai, China) preequilibrated with binding buffer (same composition as the lysis buffer). A washing buffer (binding buffer with the addition of 30 mM imidazole) was used to remove impurities, and proteins were then eluted (binding buffer with 500 mM imidazole). Proteins were further purified by size exclusion chromatography using a HiLoad 16/600 Superdex 75 column (GE Healthcare, Shanghai, China).

The TDRD3 Tudor domain (555-608), TTC (the construct was synthesized by Tsingke Biotechnology Co., Ltd., Nanjing, China), and associated mutants were expressed and purified as described above.

The MBP-PRMT1 vector containing an ampicillin resistance cassette was cotransformed with the pET-28a G3BP1 RRM-RGG vector. The steps for purification of the methylated RRM-RGG were the same as those for the wild type. The Mass spectrometry were used to check the methylation status of G3BP1 RRM-RGG.

The total RNAs were extracted from Hela cells by TRIZOL method.

For NMR experiments, bacteria were cultured in a minimal media (24g NaH_2PO_4, 5g NaOH, 0.5g NH_4Cl, and 2.5g glucose in 1 L H2O), where NH_4Cl was isotope labeled. The expression and purification of proteins were described above.

Synthesis of the peptides

The peptides:

R447^{me2a}: GPPR^{me2a}GGMVQK,

R435^{me2a}: GPR^{me2a}GG,

R435^{me2a}/R447^{me2a}: GPR^{me2a}GGLGGGMRGPPR^{me2a}GGMVQK,

unmodified: GPRGGLGGGMRGPPRGGMVQK

were synthesized by Tsingke Biotechnology Co., Ltd. (Nanjing, China).

NMR spectroscopy

For the TDRD3 Tudor domain (555-608), proteins were dialyzed against PBS buffer (150 mM NaCl and 20 mM NaH_2PO_4, pH 6.5). The data were processed and analyzed by NMRPipe and SPARKY[1, 2]. The ^{15}N-labeled proteins (0.1 mM) were used in the acquisition of 2D ^{1}H-^{15}N HSQC spectra on an Agilent 500 MHz or 700 MHz system at a series of ligand: protein molar ratios. The backbone chemical shift assignment of TDRD3 Tudor domain was retrieved from BMRB (BMRB Entry:18490). The chemical shift change of each peak was calculated as:

$$\Delta = \sqrt{\frac{\Delta_H^2 + 0.04\Delta_N^2}{2}}$$

where Δ_H and Δ_N were the chemical shift changes of each peak in the ^{1}H and ^{15}N

dimension, respectively. The protein-ligand binding affinity was best fitted from the dose-dependent chemical shift changes by the following equation[3],

$$\Delta = \frac{\Delta_m\left[(P_0 + L_0 + K_d) - \sqrt{(P_0 + L_0 + K_d)^2 - 4P_0L_0}\right]}{2P_0}$$

where the P_0 and L_0 represent the protein and ligand concentration, respectively. Δ_m represents the maximum chemical shift changes upon saturation. The global fitting of residues of significant chemical shift changes was applied with a shared K_d value.

Liquid-liquid phase separation and fluorescence recovery after photobleaching (FRAP) assays

All proteins, RNAs, and peptides were dissolved or purified in PBS buffer (150 mM NaCl and 20 mM NaH_2PO_4, pH 6.5). An OLYMPUS-IX81 or LSM710 (Zeiss) microscope was used to acquire LLPS images. For the FRAP assay, Alexa Fluor 488 was conjugated to the N-termini of proteins. Each of the components for LLPS was quickly mixed in a volume of 5 μL, and images were then acquired. All of these procedures were performed within 10 min. Images were processed by ImageJ.

For FRAP, the fluorescence intensity of droplets was bleached at least 50%, and the recovery of fluorescence was monitored at intervals of 1 second. The data were processed, and the curves were fit by Origin[4, 5].

Quantification of the LLPS or SG area ratio

The LLPS or stress granule area ratio R can be calculated as

$$R = \frac{A_g}{A_t}$$

where A_g is the area of the granule and A_t is the total area of the image or the cytoplasm[6-10]. All areas were calculated as numbers of pixels.

ell culture, transfection, and arsenite treatment

U2OS cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified incubator containing 5% CO_2. Cells were transfected with plasmid DNA using Lipofectamine 3000 (Thermo Fisher Scientific, Shanghai, China). For arsenite treatment, cells were placed in fresh medium for 1 h and stressed with 500 μM sodium arsenite at 37°C.

Antibodies

The anti-TDRD3 antibody was purchased from Proteintech (Wuhan, China, 13359-1-AP, 1:1000 dilution for WB) and the anti-G3BP1 antibody was purchased from Sigma–Aldrich (Shanghai, China, PLA0231).

Imaging of stress granules in cells

For the immunofluorescence study, cells were cultured on 20 mm×20 mm glass coverslips coated with poly-D-lysine (Sigma–Aldrich, Shanghai, China) and placed in 35 mm dishes (Wuxi NEST Biotechnology Co., Ltd., Wuxi, China). Transfected cells were sequentially fixed with 4% paraformaldehyde (Electron Microscopy Science, Beijing, China) in PBS for 10 min, permeabilized with 0.4% Triton X-100 in PBS for 10 min, and blocked with 1% BSA for 30 min, with all steps performed at room temperature. Samples were further incubated with primary antibodies in a blocking

buffer overnight at 4 °C, washed 3 times with PBST (0.1% Tween), and incubated with a secondary antibody for 1 h at room temperature. Then, 4,6-diamidino-2-phenylindole (DAPI) was used to visualize nuclei. The primary antibodies used in this study are listed in the Key Resources Table. Host-specific Alexa Fluor 488/594-conjugated secondary antibodies (Proteintech, Wuhan, China) were used for visualization. Images were acquired with a Nikon Eclipse Ti-E microscope.

Cell viability assay

U2OS cells were seeded in triplicates in 96-well plates at 10000 cells/well. After being treated with AS or **BiTud** for 2h, cell viability was assessed by CCK-8 according to the manufacturer's instructions. Briefly, Cell culture media were changed into fresh media containing 10% (v/v) CCK-8 reagent at indicated times. After a 1h incubation under the culture condition, the absorbance at 450 nm of each well was measured on a microplate reader. The mean value of the wells with media alone was used as background and was subtracted from the absorbances of the wells containing cells.

Colony formation assay

U2OS cells were plated into a twelve-well plate at 100000 cells/well and treated with AS or TDRD3 inhibitor after adhesion. Following 2 days of incubation, the colonies were fixed with 4% paraformaldehyde for 30 min and stained with 1% crystal violet for 2 h at room temperature. The plates were then washed and dried before photographic images were captured.

Synthesis of the bivalent inhibitor molecule

Dibromide (512 mg, 2 mmol) and K_2CO_3 (830 mg, 6 mmol) were added to a solution of N-aryl piperidine[1] (1.2 g, 5 mmol) in DMF (33 mL). The reaction mixture was stirred at 50 °C for 24 h. After cooling to room temperature, the reaction mixture was diluted with EtOAc, washed with water 3 times, and then washed with brine. The separated organic phase was dried over anhydrous Na_2SO_4, and the solvent was evaporated under a vacuum. The residue was purified by flash column chromatography to give the desired product (272 mg, 32% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.73 (d, J = 2.5 Hz, 2H), 7.40 (dd, J = 8.8, 2.5 Hz, 2H), 7.06 (d, J = 8.8 Hz, 2H), 3.69–3.54 (m, 12H), 3.11–2.99 (m, 8H), 2.71–2.60 (m, 12H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 144.8, 143.2, 133.5, 126.7, 125.8, 122.3, 70.7, 70.5, 68.9, 57.7, 53.4, 51.6. HRMS (ESI): m/z calculated for $C_{28}H_{39}Cl_2N_6O_7$ ($[M+H]^+$): 641.2252, found: 641.2273.

Mass spectrometry

Digestion was performed for G3BP1 methylation site analysis. The peptide mixture was analyzed with an in-house 30 cm-long pulled-tip analytical column (75 μm ID packed with ReproSil-Pur C18-AQ 1.9 μm resin, Dr. Maisch GmbH), and the column was then connected in line to a nanoElute HPLC (Bruker Daltonics) for mass spectrometry analysis. The analytical column temperature was set to 55 ℃ during the experiments. The mobile phase used for peptide separation was 0.1% formic acid in water as buffer A and 0.1% formic acid in 100% acetonitrile as buffer B, and the elution gradient was as follows: 0-90 min, 2%-22% B; 90-100 min, 22%-37% B; 100-110 min, 37%-95% B; 110-120 min, 95% B. The flow rate was set to 300 nL/min.

Data-dependent MS/MS analysis was performed with a timsTOF Pro mass spectrometer (Bruker Daltonics). For G3BP1 methylation site analysis, the acquired

MS/MS data were matched against the UniProtKB *Homo sapiens* database (released on Aug. 14, 2020) using PEAKS Online X. Cysteine alkylation by iodoacetamide was specified as the fixed modification with a mass shift of 57.02146, and methionine oxidation was set as the dynamic modification with a mass shift of 15.9949. Additionally, lysine and arginine mono/dimethylation were set as variable modifications with a mass shift of 14.0156 and 28.0313, respectively.

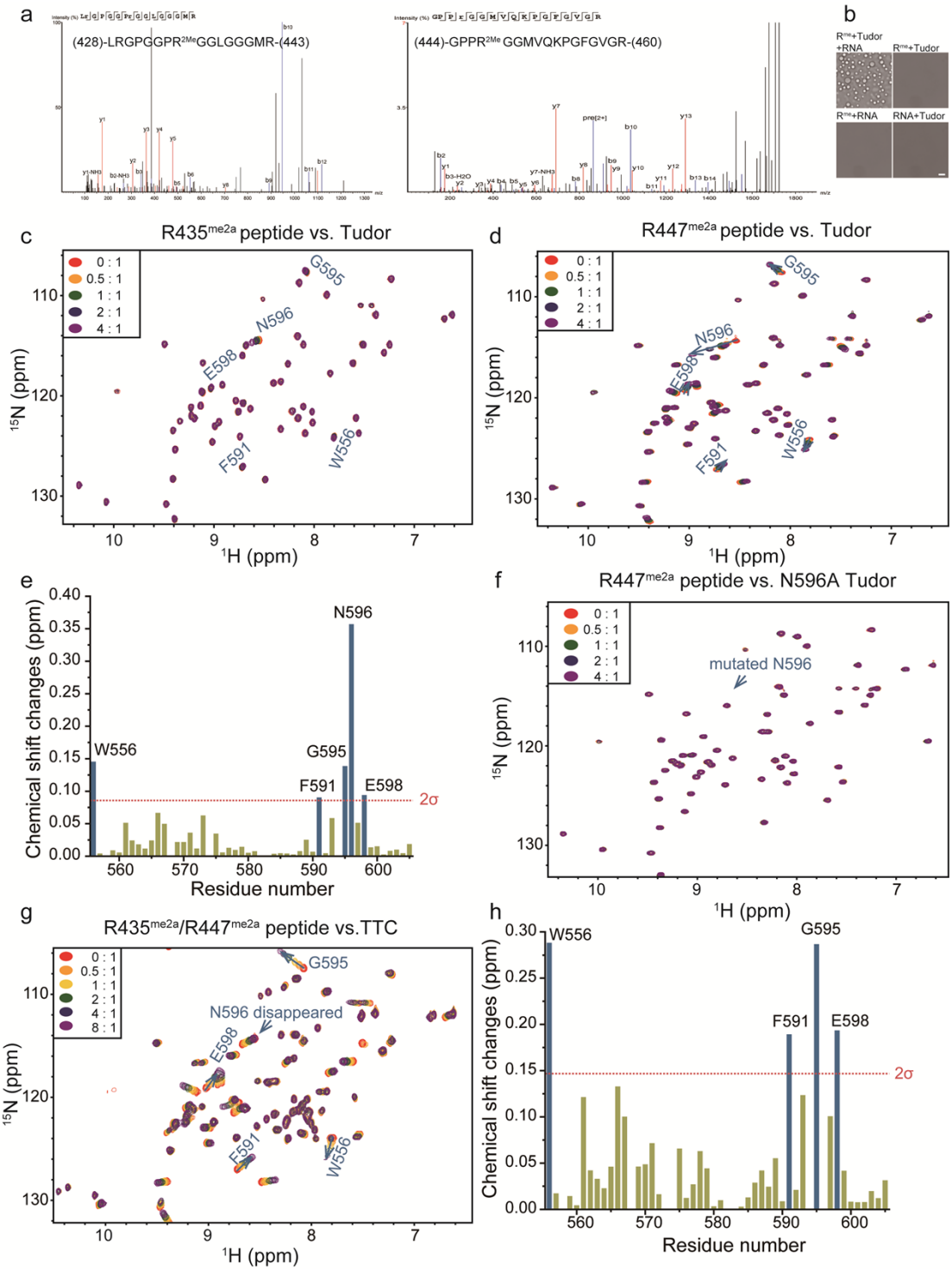

Fig. S1 NMR HSQC spectra of ^{15}N-labeled TDRD3 Tudor chimeras upon titration of different methylated G3BP1 peptides. (a)R435 (left) and R447 (right) of purified G3BP1 RRM-RGG (R^{me}) were detected to be di-methylated by co-expressed PRMT1. (b) The mixture of methylated G3BP1 RRM-RGG (R^{me}), TDRD3 Tudor and RNAs two-by-two or together. Imagines are captured by OLYMPUS-IX81, R^{me}: 50 μM. Tudor: 50 μM. RNA: 20 ng/μL. scale bar: 10 μm. (c-d) NMR titrations of different peptides to TDRD3 Tudor. Annotated are the compound/protein molar ratios. Tudor: 100 μM (e) Chemical shift changes of TDRD3 Tudor when R447^{me2a} peptide titrated to

Tudor at the molar ratio of 4. Mean+2*SD (2σ, dash line) were calculated including all peaks but only assigned peaks were demonstrated as bar chart. (f) Titration of R447^{me2a} peptide to TDRD3 N596A mutant. Protein: 100 μM. (g) NMR titrations of different peptides to TDRD3 TTC. Annotated are the compound/protein molar ratios. TTC: 100 μM (h) Chemical shift changes of TTC when R435^{me2a}/R447^{me2a} peptide titrated to TTC at the molar ratio of 8.

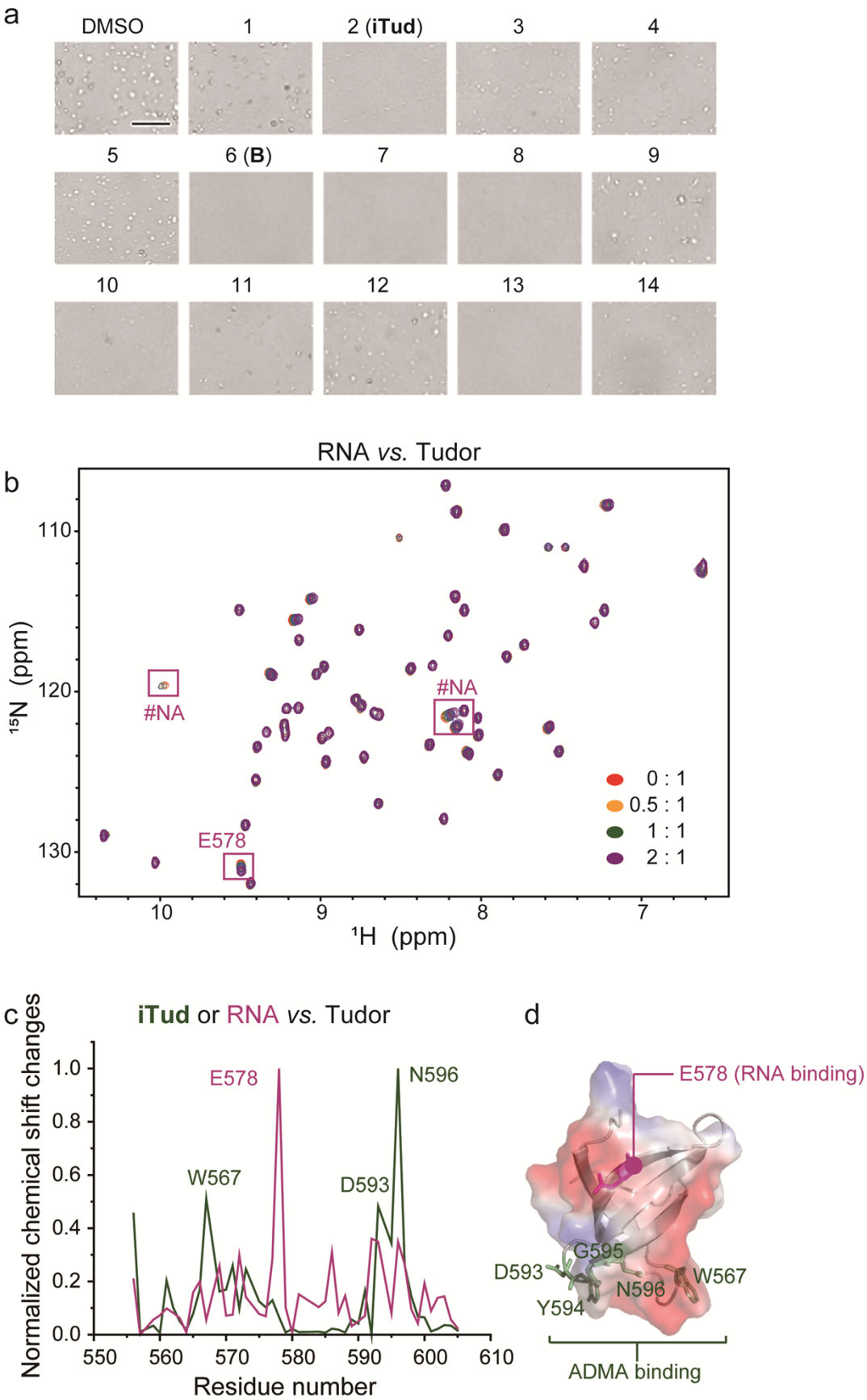

Fig. S2 Screening of the inhibitors and the bridging role of TDRD3 Tudor uncovered by the inhibitor. (a) Screening of 14 inhibitors of TDRD3 Tudor domain

using LLPS assay of the three-component methylated G3BP1-TDRD3-RNA system. Imagines are captured by OLYMPUS-IX81, R^{me}: 50 μM. Tudor: 50 μM. RNA: 20 ng/μL. scale bar, 20 μm. (b) Titration of 20nt poly U to TDRD3 Tudor domain saturated by 5-folded **iTud**. Tudor: 100 μM. The #NA represents the residue not assigned. (c) Analysis of chemical shift changes caused by **iTud** and RNAs. (d) Mapping of the mostly perturbated residues to the structure of TDRD3 Tudor (PDBID: 5yj8).

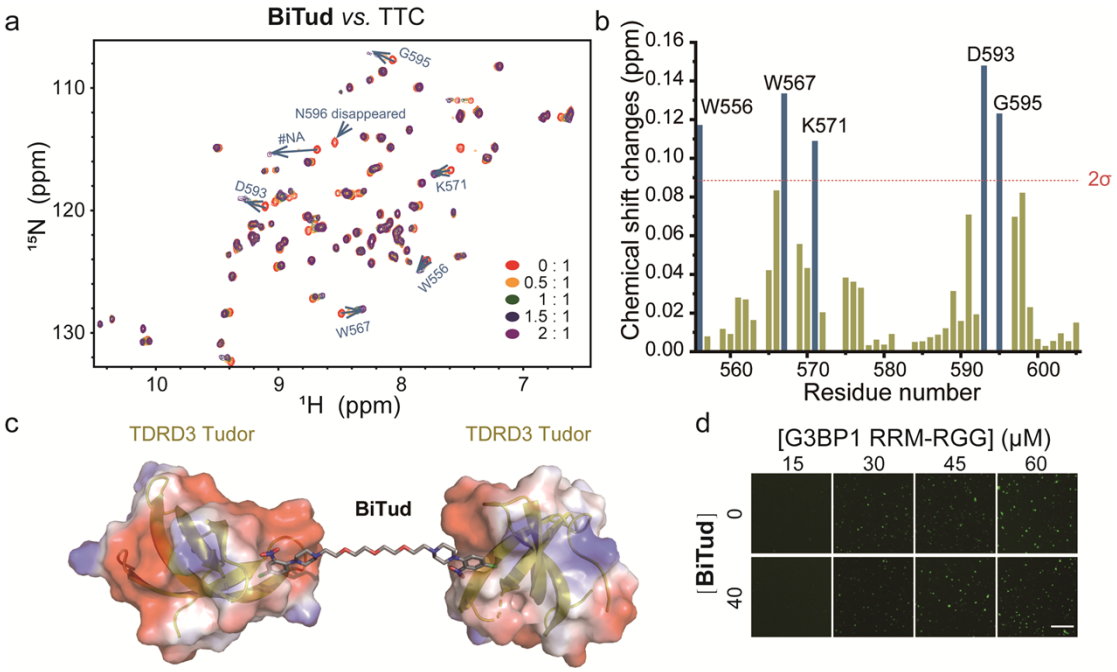

Fig. S3 Validation of the inhibiting ability of BiTud *in vitro*. (a-b) Superimposition of NMR HSQC spectra and chemical shift changes of ^{15}N-labeled tandem Tudor chimeras upon titration of the bivalent inhibitor **BiTud**. Annotated are the compound/protein molar ratios. TTC: 20 μM. The #NA represents the residue not assigned. (c) Docking model of **BiTud** with two TDRD3 Tudor domain given by Smina. The ChimeraX was used for the conversion of coordinates. (d) **BiTud** did not suppress the LLPS of unmethylated G3BP1 RRM-RGG and RNAs. Scale bar: 10 μm. RNA: 20 ng/μL.

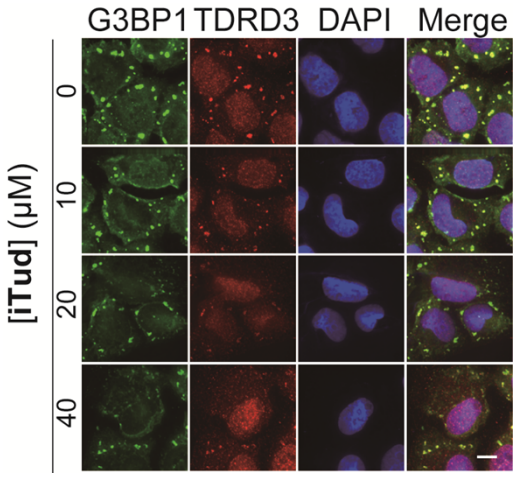

Fig. S4 Inhibitors suppress the growth of SGs. U2OS cells were incubated with **iTud** before arsenite treatment (500 μM) for 1 h. Cells were then fixed and stained with DAPI (blue), an anti-TDRD3 antibody (green), and an anti-G3BP1 antibody (red). Images were acquired with a Nikon Eclipse Ti-E. Scale bar, 10 μm.

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